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Award Number: DAMD17-00-1-0333

TITLE: In Vivo Structure-Function Studies on the Precise Role of ErbB-2 Signaling in Mammary Gland Development

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REPORT DATE: October 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20030317 031

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED	
	October 2002	Annual Summary (1 Sep 01 - 1 Sep 02)	
4. TITLE AND SUBTITLE In Vivo Structure-Function Studies on the Precise Role of ErbB-2 Signaling in Mammary Gland Development			5. FUNDING NUMBERS DAMD17-00-1-0333
6. AUTHOR(S) Richard Chan Dr. William Muller			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) McMaster University Hamilton, Ontario, Canada, L8S 4K1 E-Mail: chanr@mcmaster.ca			8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES Original contains color plates: All DTIC reproductions will be in black and white.			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The ErbB-2/Neu receptor tyrosine kinase plays important roles in development and disease. To genetically dissect the ErbB-2 mediated signaling pathway <i>in vivo</i> , we have generated <i>neu</i> cDNA knock-in mutants harboring subtle mutations and expressed under the transcriptional control of the endogenous promoter. We have shown that the kinase activity of ErbB-2 is essential for embryonic development since this mutant is a phenocopy of the <i>erbB-2</i> null mutants. We also demonstrate that the loss of tyrosine 1028 results in a hypermorphic phenotype in animals, strongly suggesting that Y1028 does indeed convey a negative regulatory signal on ErbB-2. The mechanism for this action is under investigation. However, we investigated the possibility that c-Cbl was mediating this downregulatory action by interacting with Y1028 and targeting ErbB-2 for ubiquitylation and degradation. Although we did not observe c-Cbl association with Y1028, we did identify c-Cbl association with two other tyrosine phosphorylation sites, Y1144 and Y1227. The nature and significance of this interaction is currently under investigation. The identity of signaling events that may lead to the downregulation of ErbB-2 would have important therapeutic and clinical implications.			
14. SUBJECT TERMS Epidermal Growth Factor receptor, ErbB-2/Neu, Tyrosine Kinase, Phosphorylation, Targeted Knock-in, c-Cbl			15. NUMBER OF PAGES 17
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

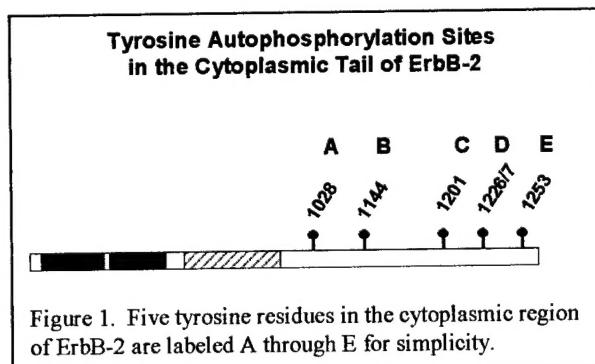
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Introduction

The ErbB-2/Neu receptor tyrosine kinase is a member of the Epidermal Growth Factor Receptor family of receptor tyrosine kinases (Yarden 1988). The potency of these receptors is demonstrated by their causative roles in human cancers due to aberrant activation. Furthermore, the embryonic lethality observed in animals with a targeted mutation of the gene expressing either of these receptors clearly demonstrates their critical roles in mammalian development (Lee 1995; Britsch 1998; Meyer 1995; Gassmann 1995). Thus, it is desirable to understand the diversity and specificity in receptor tyrosine kinase signaling. The ability of the receptors to form different combinations of homodimers and heterodimers contribute to this family's complex array of signaling potential (Pinkas-Kramarski 1996; Pinkas-Kramarski 1998).

The ErbB-2 receptor is thought to be the key member of this family particularly because of its potent tyrosine kinase activity. It is also considered the preferred heterodimerization partner for the other ErbB receptors forming stable and strong signaling complexes (Graus-Porta 1997). The lab of Dr. Muller has previously identified and characterized five distinct tyrosine autophosphorylation sites in the cytoplasmic tail of the receptor (Figure 1) (Dankort 1997). These phosphorylated tyrosine residues become sites of interaction with downstream signaling molecules, thereby initiating the signal transduction pathway. *In vitro* transformation assays were used to study various ErbB-2 mutants in an attempt to genetically dissect ErbB-2 receptor signaling (Dankort 1997). In my research project, I am



examining the *in vivo* effects of expressing these mutants by generating and characterizing various *erbB-2* knock-in mutants.

Body

In general, the structure of the ErbB receptors include an extracellular ligand binding domain, a single pass transmembrane domain, a tyrosine kinase domain, and a cytoplasmic tail with several distinct tyrosine phosphorylation sites. Upon activation of the receptor and phosphorylation of its tyrosine residues, these phosphotyrosine sites may then recruit various intracellular substrates to initiate signal transduction. Five distinct tyrosine phosphorylation sites in the cytoplasmic tail of ErbB-2 (Figure 1) have been identified and characterized previously by Dankort *et al.* (1997). Four of these sites (B-E) transduce redundant, but not necessarily overlapping, positive signals with respect to ErbB-2 mediated transformation whereas the remaining phosphorylation site (site A) mediates a negative regulatory signal that inhibits transformation via an unknown pathway (Dankort 1997).

We have generated several *erbB-2* knock-in mutants and are in the midst of characterizing or have completed the analysis of these knock-in mutants. The first mutant analyzed addressed the importance of the ErbB-2 kinase activity within the array of receptor hetero- and homo-dimerization. The kinase activity of ErbB-2 is thought to be a critical component of the EGFR family's signaling potential. We have addressed the significance of this kinase activity by generating mice expressing a kinase-dead ErbB-2 (Neu-KD) receptor. This analysis is complete and was published in Molecular and Cellular Biology Volume 22, p. 1073-1078, 2002 (Appendix 1). Briefly, we demonstrated that the kinase-dead ErbB-2 receptor acts as a functionally null receptor *in vivo* and results in a phenotype identical to the *erbB-2* null mice. These mice die at midgestation due to aberrant heart development and

display abnormal peripheral nervous system development (Lee 1995; Britsch 1998). Thus, we concluded that the kinase activity of ErbB-2 is absolutely required for ErbB-mediated signaling.

To examine the role of the cytoplasmic tail of ErbB-2 and its tyrosine phosphorylation sites, *in vivo*, we have generated a targeting construct to create knock-in mice expressing the Neu tYrosine Phosphorylation Deficient (NYPD) receptor. This NYPD mutant is an ErbB-2 receptor where the five tyrosine autophosphorylation sites have been mutated to phenylalanine residues. *In vitro*, this mutant receptor is greatly impaired in its ability to transform fibroblast cells (Dankort 1997). This construct was electroporated into R1 ES cells and chimeric animals were generated. However, we encountered unresolved problems with the chimeras and the animals had to be sacrificed.

We have also previously generated knock-in mice expressing either the wild-type *neuN* cDNA or a phosphorylation mutant *neu* cDNA and are currently characterizing these animals. Wild-type *neuN* knock-in animals are apparently normal, healthy and fertile. However, upon closer examination of the protein levels in E12.5 *neuN* knock-in embryos, we noticed that the expression of Neu was only about 10% of the endogenous levels in a wild-type animal (Figure 2). This is likely due to the nature of the knock-in design. However, in knock-in animals expressing the Y1028F point mutant (site A), the level of expression was significantly higher than that of the *neuN* knock-in allele at about 30-50% of endogenous wild-type levels (Figure 2). To examine the consequences of further reducing the level of Neu in the knock-in animals, we introduced the *erbB-2* null allele such that animals only

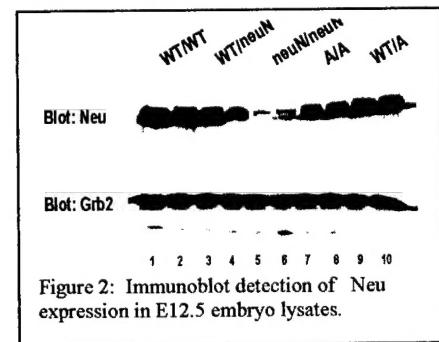


Figure 2: Immunoblot detection of Neu expression in E12.5 embryo lysates.

possessed a single *erbB-2/neu* allele. These animals died shortly after birth due to an inability of the newborn pups to inflate their lungs (Figure 3). Knock-in mice expressing a point mutation at tyrosine 1028 (site A) of the ErbB-2 receptor were generated using the same targeting strategy. These mice were also normal, healthy, and fertile. Interestingly, when the *erbB-2* null allele was introduced, the presence of the Y1028F mutation completely rescued the neonatal lethality seen with the wild-type *neuN* knock-in animals described above. Thus, we concluded that tyrosine 1028 acts as a negative regulator of ErbB-2 receptor activity and this is evident our *in vivo* model as well as *in vitro*.

We investigated whether the downregulatory mechanism associated with Y1028 was mediated by the protein c-Cbl. c-Cbl is a multi-adaptor protein that can regulate the activity of receptor tyrosine kinases by recruiting the ubiquitin machinery and promoting the multi-ubiquitylation of activated receptors (reviewed by Thien and Langdon, 2001). Poly-ubiquitylation targets the receptors for proteosomal degradation and thereby suppresses receptor signaling (Weissmann 2001). Thus, c-Cbl functions to attenuate activated receptor tyrosine kinases by promoting the downregulation and degradation of the receptor. The importance of c-Cbl association with other receptor tyrosine kinases such as the EGFR (Levkowitz et al., 1998), MET (Peschard et al., 2001), and PDGFR (Miyake et al., 1999) has been demonstrated recently.

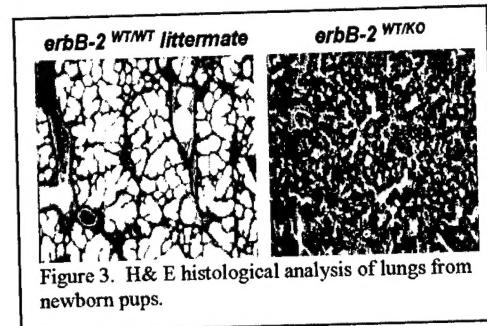


Figure 3. H&E histological analysis of lungs from newborn pups.

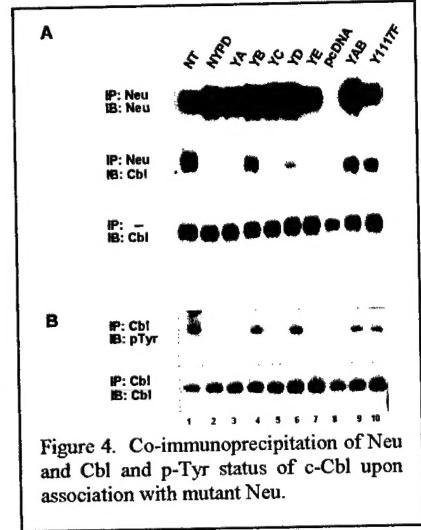


Figure 4. Co-immunoprecipitation of Neu and Cbl and p-Tyr status of c-Cbl upon association with mutant Neu.

Co-expression of various Neu mutants and c-Cbl in 293T cells demonstrated that c-Cbl does indeed interact with Neu but this association is not dependent on Y1028 (Figure 4A). The ubiquitylation status of Neu is also not dependent on Y1028 (Figure 5). Thus, we are currently investigating the exact mechanism of Y1028 mediated downregulation of Neu by examining protein stability and turnover in stable cell lines. We will also be using proteomics technology to identify proteins that may interact with Y1028 to mediate the negative regulatory effects.

We have identified that c-Cbl association with Neu is via and Y1144 (YB) and Y1227 (YD) (Figure 4A) and that this interaction correlates with the tyrosine phosphorylation of c-Cbl (Figure 4B). Interestingly, both of these sites also interact directly (Y1144) or indirectly (Y1227) with the Grb2 adaptor protein, which is known to associate with c-Cbl (Fukazawa 1995). However, upon investigation of the ubiquitylation status of Neu, unlike the other receptor tyrosine kinases, c-Cbl association with Neu did not correlate with the ubiquitylation of the receptor (Figure 5). In fact, ubiquitylation of Neu is independent of c-Cbl association. Upon further investigation, we have also demonstrated that the ubiquitylation of Neu is dependent on the phosphorylation of Y1253 (YE) (Figure 5 and 6) but this does not appear to correlate with any downregulation or degradation of the receptor.

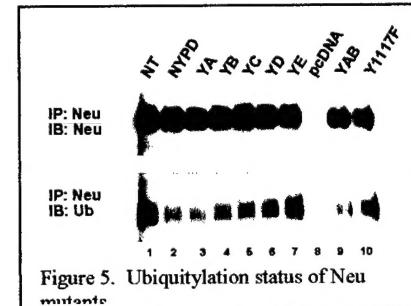


Figure 5. Ubiquitylation status of Neu mutants

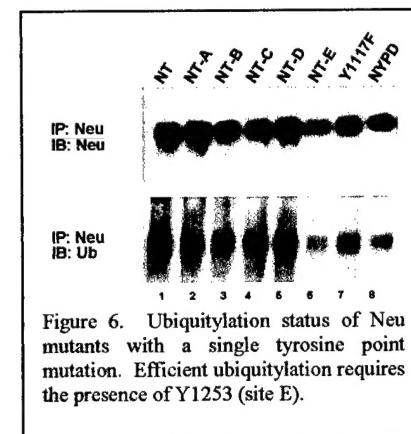


Figure 6. Ubiquitylation status of Neu mutants with a single tyrosine point mutation. Efficient ubiquitylation requires the presence of Y1253 (site E).

Key Research Accomplishments:

- Completed analysis of mice expressing a kinase-dead ErbB-2 receptor.
- Results published in Molecular and Cellular Biology (Appendix 1).
- Generated and started characterizing mutant *neu* cDNA knock-in mice.
- Identified an *in vivo* negative regulatory function of Neu Y1028 via increased protein stability – however, the exact mechanism is still under investigation.
- Identified c-Cbl association with Neu at two tyrosine phosphorylation sites (Y1144 and Y1227).
- Identified a tyrosine phosphorylation site (Y1253) that is required for efficient ubiquitylation of Neu.

Reportable Outcomes:

- Publication in Molecular and Cellular Biology 22: p1073-1078 (2002): “*The catalytic activity of the ErbB-2 receptor tyrosine kinase is essential for embryonic development*”

Conclusions:

In summary, we have generated an *in vivo* knock-in system to perform structure-function analysis on the ErbB-2/Neu receptor. These analyses lead us to the investigation of a tyrosine phosphorylation site (Y1028) that negatively regulates the activity of the receptor, presumably through stabilization of the receptor. The precise mechanism is currently under study. We have also identified c-Cbl association with ErbB-2 (Y1144 and Y1227), however, this association does not lead to receptor ubiquitylation and downregulation, as seen with other receptor tyrosine kinases. The exact nature and role of c-Cbl association with ErbB-2 (via Grb2) and why ErbB-2 is resistant to c-Cbl mediated ubiquitylation is currently being investigated. c-Cbl is a multi-adaptor protein that can also mediate positive

signals, thus, we will determine the effects of c-Cbl downstream of ErbB-2 mediated signaling. To study the role of the ErbB-2/c-Cbl interaction in mammary gland development and tumorigenesis, we will introduce the MMTV-NDL (activated Neu) (Siegel 1999) into a *c-cbl* deficient background. *c-cbl* deficient mice display hyperplastic ductal development, suggesting a potential biological association with ErbB family receptors in the mammary gland (Murphy et al., 1998). We will also compare in our knock-in mice if the hypomorphic expression of Neu affects the mammary gland.

The downregulation of receptor tyrosine kinases is an important concept to study and understand because of its obvious clinical implications in treating cancers due to aberrant activation of receptor tyrosine kinases. The ability to manipulate natural mechanisms of downregulating receptors may result in more precise targets with fewer or minimal side effects.

References

Britsch, S., L. Li, S. Kirchhoff, F. Theuring, V. Brinkmann, C. Birchmeier, and D. Riethmacher. 1998. The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes Dev* **12**:1825-1836.

Dankort, D.L., Wang, Z., Blackmore, V., Moran, M.F., and Muller, W.J. 1997. Distinct tyrosine autophosphorylation sites negatively and positively modulate Neu-mediated transformation. *Mol. Cell. Biol.* **17**: 5410-5425.

Fukazawa T, Reedquist KA, Trub T, Soltoff S, Panchamoorthy G, Druker B, Cantley L, Shoelson SE, Band H. The SH3 domain-binding T cell tyrosyl phosphoprotein p120. Demonstration of its identity with the *c-cbl* protooncogene product and in vivo complexes with Fyn, Grb2, and phosphatidylinositol 3-kinase. *J Biol Chem.* 1995 **270**:19141-50.

Gassmann, M, F. Casagranda, D. Orioli, H. Simon, C. Lai, R. Klein, G. Lemke. 1995. Aberrant neural and cardiac development in mice lacking the erbB4 neuregulin receptor. *Nature* **378**:390-394.

Graus-Porta, D., R.R. Beerli, J.M. Daly, and N.E. Hynes. 1997. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J* **16**:1647-1655.

Lee, K.F., H. Simon, H. Chen, B. Bates, M.C. Hung, and C. Hauser. 1995. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**: 394-398.

Levkowitz G, Waterman H, Zamir E, Kam Z, Oved S, Langdon WY, Beguinot L, Geiger B, Yarden Y. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev.* 1998 **12**:3663-74.

Meyer D. and C. Birchmeier. 1995. Multiple essential functions of neuregulin in development. *Nature* **378**: 386-390.

Miyake S, Mullane-Robinson KP, Lill NL, Douillard P, Band H. Cbl-mediated negative regulation of platelet-derived growth factor receptor-dependent cell proliferation. A critical role for Cbl tyrosine kinase-binding domain. *J Biol Chem.* 1999 **274**:16619-28.

Murphy, M.A., R.G. Schnall, D.J. Venter, L. Barnett, I. Bertoncello, C.B.F. Thien, W.Y. Langdon, D.D. Bowtell. 1998. Tissue Hyperplasia and Enhanced T-cell Signalling via ZAP-70 in c-Cbl Deficient Mice. *Mol Cell Biol* **18**:4872-4882.

Peschard P, Fournier TM, Lamorte L, Naujokas MA, Band H, Langdon WY, Park M. Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. *Mol Cell.* 2001 **8**:995-1004.

Pinkas-Kramarski, R., M. Shelly, B.C. Guarino, L.M. Wang, L. Lyass, I. Alroy, M. Alimandi, A. Kuo, J.D. Moyer, S. Lavi, M. Eisenstein, B.J. Ratzkin, R. Seger, S.S. Bacus, J.H. Pierce, G.C. Andrews, Y. Yarden, and M. Alamandi. 1998. ErbB tyrosine kinases and the two neuregulin families constitute a ligand-receptor network (published erratum appears in *Mol Cell Biol* 1998 Dec;18(12):7602). *Mol Cell Biol* **18**:6090-6101.

Pinkas-Kramarski, R., L. Soussan, H. Waterman, G. Levkowitz, I. Alroy, L. Klapper, S. Lavi, R. Seger, B.J. Ratzkin, M. Sela, and Y. Yarden. 1996. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J* **15**:2452-2467.

Siegel PM, Ryan ED, Cardiff RD, Muller WJ. Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. *EMBO J.* 1999 **18**:2149-64.

Thien CB, Langdon WY. Cbl: many adaptations to regulate protein tyrosine kinases. *Nat Rev Mol Cell Biol.* 2001 **2**:294-307.

Weissman AM. Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol.* 2001 **2**:169-78.

Yarden, Y. and A. Ullrich. 1988. Growth factor receptor tyrosine kinases. *Annu Rev Biochem* **57**:443-478.

The Catalytic Activity of the ErbB-2 Receptor Tyrosine Kinase Is Essential for Embryonic Development

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Received 17 September 2001/Returned for modification 25 October 2001/Accepted 2 November 2001

Activation of the epidermal growth factor receptor (EGFR) family is thought to play a critical role in both embryogenesis and oncogenesis. The diverse biological activities of the EGFR family are achieved through various ligand-receptor and receptor-receptor interactions. One receptor that has been found to play a central role in this signaling network is ErbB-2/Neu, and it is considered the preferred heterodimerization partner for other members of the EGFR family. To assess the importance of the catalytic activity of ErbB-2 in embryonic development, we have generated mice expressing a kinase-dead erbB-2 cDNA under the transcriptional control of the endogenous promoter. Here, we show that mice homozygous for the kinase-dead erbB-2 allele die at midgestation and display the same spectrum of embryonic defects seen in erbB-2 knockout mutants. These observations suggest that the catalytic activity of ErbB-2 is essential for normal embryonic development.

The epidermal growth factor receptor (EGFR) family of growth factor receptor tyrosine kinases, including ErbB-1/EGFR, ErbB-2/Neu, ErbB-3, and ErbB-4, have been implicated in breast cancer as well as several other human cancers (2). Recently, gene targeting studies have demonstrated specific roles for each of the EGFR family members in normal mammalian development. For example, *erbB-2* (12) and *erbB-4* (6) knockout mice die at midgestation due to deficient cardiac function associated with a lack of myocardial ventricular trabeculation and display abnormal development of the peripheral nervous system. Cardiac rescue of the defects seen in *erbB-2* null mice revealed additional roles for ErbB-2 at the developing neuromuscular junction (13, 17). ErbB-3 mutant mice have less severe defects in the heart and consequently are able to survive several days later through embryogenesis. However, sensory and motor neurons in these animals show signs of degeneration due to a lack of proper Schwann cell development (1, 22).

Although the structures of the EGFR family receptors have been described in detail, their individual roles in and contributions to these developmental processes remain to be investigated. To achieve the observed diversity in signaling potential, a coordinate array of ligand-receptor and receptor-receptor interactions are possible. Following activation of the various EGFR family members with one of several EGF family ligands, both homodimeric and heterodimeric combinations of receptors are induced, and their intrinsic catalytic tyrosine kinase activity is stimulated (30). Upon receptor dimerization, specific tyrosine residues residing in the terminal tail of the receptor dimer become phosphorylated and serve as important potential binding sites for various intracellular signaling proteins.

Although activated ErbB receptors may partake in any particular combination of homodimerization or heterodimerization complexes, it is important to note that a hierarchical order of preference, stability, and signaling potential for each receptor combination is in effect (7). In particular, there is generally a greater preference and likely an advantage for dimerization complexes to include ErbB-2 as the partner because of its potent intrinsic kinase activity. Since no identified ligand is known to bind to and activate ErbB-2 alone, it is considered an orphan receptor. Thus, stimulation of ErbB-2 kinase activity may be mediated through normal ligand activation of another ErbB receptor first, which subsequently engages in a specific heterodimer complex (3, 18, 19). In contrast, ErbB-3, which can bind to and become activated by the ligand neuregulin, is naturally kinase inactive and therefore must depend on a heterodimerization partner for phosphorylation of its tyrosine residues (8, 26). Indeed, the ErbB-2-ErbB-3 complex is very stable and transmits a strong mitogenic signal (11). These observations strongly suggest that ErbB-2 plays a central role in the EGFR family signal transduction.

Although genetic ablation studies demonstrate the importance of a receptor to a biological function, they do not address precisely how and which of the individual functional domains of the receptor contribute to the phenotype. It is also unclear within this complex array of receptor dimerization whether the loss of ErbB-2 results in direct or indirect consequences of a lack of the receptor and its interactions with other proteins.

To assess exactly how ErbB-2 may act as the central mediator of the EGFR family of receptors, we investigated whether the kinase activity of ErbB-2 is indeed essential for its complete biological effects. To accomplish this, we generated mice expressing a kinase-dead *erbB-2* cDNA under the transcriptional control of the endogenous *erbB-2* promoter. Mice homozygous for the kinase-dead *erbB-2* mutation died at embryonic day 10.5 (E10.5) due to a lack of cardiac trabeculation and displayed defects in neural development. These observations argue that the catalytic activity of ErbB-2 is absolutely required

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for normal embryonic development and cannot be compensated for by other members of the EGFR family.

MATERIALS AND METHODS

Generation of targeting construct and mutant mice. Oligonucleotide-directed PCR mutagenesis with the following primers was used to create the mutant (K757 M) kinase-dead ErbB-2 receptor: AB11151, GGA AGT ATA CGA TCG CTA GGC; AB10015, CAC CAT GAT AGC CAC GGG GAT TTT CAC; AB 10014, C GTG GCT ATC ATG GTG TTG AGA GAA AAC; and AB 11152, CGA CCT CGG TGT TCT CGG AC. The underlined nucleotides identify the sequence substituted to create the desired mutation. PCR products were subsequently subcloned into a wild-type *erbB-2* cDNA and then cloned into the final targeting vector. This plasmid was electroporated into R1 embryonic stem (ES) cells, and G418- or geneticin (Gibco-BRL)-resistant colonies were picked and subsequently screened by Southern blot analysis for correctly targeted mutants. Mutant mice were generated from the positive ES cell clones by the method of blastocyst injection into BALB/c-derived blastocysts (10). Subsequent generations were maintained in an SV129/BALB/c background.

RNase protection assays. RNA was extracted and purified from individual embryos by the guanidine isothiocyanate-cesium chloride method as previously described (27). For RNase protection assays, as described previously (24), 30 μ g of total RNA was used and hybridized to an antisense *erbB-2* riboprobe (23). The protocol was modified by lowering the overnight hybridization temperature to 45°C and only T₁ RNase (450 U) was used in the digestion reactions for 20 min at 37°C.

Western blot analysis. Fresh or flash-frozen embryos were lysed in TNE lysis buffer (24). Cleared lysates were electrophoresed through sodium dodecyl sulfate (SDS)-polyacrylamide gels, and the proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). The membranes were blocked in 10% skim milk-Tris-buffered saline (TBS) for 1 h at room temperature and then incubated with the appropriate antibody. For ErbB-2 immunoblots, the membranes were incubated with anti-ErbB-2 antibodies (1:1,000; AB-3, Oncogene Science) overnight at 4°C. Immunoblots for Grb2 were performed with rabbit anti-Grb2 polyclonal sera (1:2,500; C-23, Santa Cruz). After the primary antibody incubations, membranes were subjected to four 15-min washes in TBS-1% Tween 20 (Bio-Rad). Subsequently, horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin secondary antibodies (1:5,000; Jackson Laboratories) were incubated with the membranes for 1 h at room temperature and then washed twice for 15 min each in TBS-1% Tween-20 and twice for 15 min each in TBS alone. Immunoblots were visualized by enhanced chemiluminescence (Amersham) as specified by the manufacturer.

Histology. Embryos from timed matings were dissected free from the placenta and cleared of extraembryonic tissues. A small piece of the visceral yolk sac was retained and placed in tail lysis buffer (100 mM Tris-HCl [pH 8.5], 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μ g of proteinase K per μ l), and the DNA was isolated and subsequently used for genotyping the embryos. Dissected embryos were quickly rinsed in ice-cold phosphate-buffered saline (PBS) and transferred directly into 4% paraformaldehyde, fixed overnight at 4°C, washed twice in 70% ethanol, and stored at 4°C in 70% ethanol.

For standard hematoxylin and eosin (Fisher Scientific) staining, samples were embedded in paraffin, and 8- μ m serial sections were cut and mounted. For whole-mount *in situ* hybridizations, embryos were fixed in 4% paraformaldehyde-0.2% glutaraldehyde (Fisher Scientific), dehydrated through a graded series of methanol-PBT (1× PBS, 0.1% Tween 20; Sigma) baths and stored in 100% methanol at -20°C until needed. Whole-mount *in situ* hybridizations were carried out as previously described (29).

In vitro transcription of Phox2a riboprobes. The Phox2a riboprobe plasmid, pKS903 SSN (25), was digested with *Sst*II and transcribed with the T3 RNA polymerase to generate an antisense riboprobe; the sense riboprobe was generated using the same template but linearized with *Hind*III and transcribed with T7 RNA polymerase. The *in vitro* transcription reactions were carried out in 20- μ l reaction volumes containing 14 μ l of distilled water (diethyl pyrocarbonate [DEPC] treated), 2 μ l of 10× transcription buffer (Boehringer Mannheim), 2 μ l of DIG RNA labeling mix (Boehringer Mannheim), 1 μ g of template DNA, 30 U of RNAGuard (Pharmacia), and 30 U of RNA polymerase (Boehringer Mannheim or Gibco-BRL). The reactions were incubated at 37°C for 2 h and stopped with the addition of 20 U of DNase I (RNase-free; Boehringer Mannheim). The riboprobes were precipitated and resuspended in 100 μ l of DEPC-treated distilled water (\approx 100 ng/ μ l).

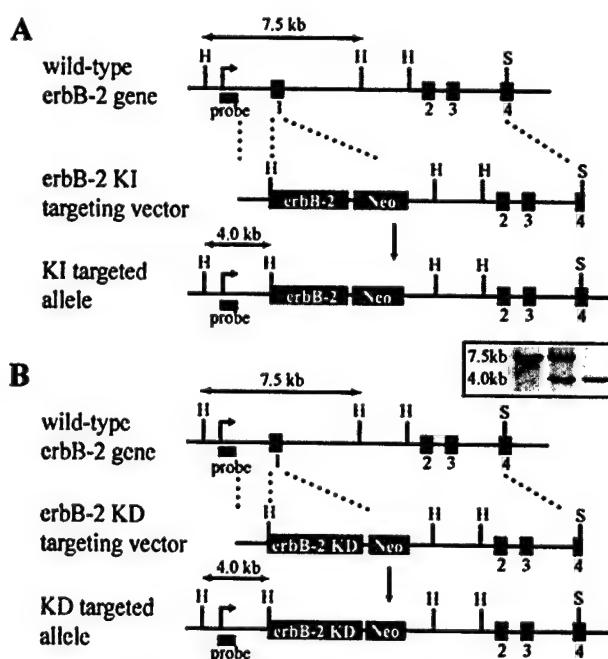


FIG. 1. Targeted *erbB-2* cDNA knock-in strategy by homologous recombination. For germ line expression, a targeting vector was constructed in which exon 1 was replaced by either (A) a wild-type *erbB-2* cDNA or (B) a cDNA encoding the kinase-dead *erbB-2* mutation, followed by a PGK-neomycin (Neo) cassette and targeted to the endogenous *erbB-2* locus by homologous 5'- and 3'-flanking arms. Digestion of genomic DNA with *Hind*III (H) and subsequent Southern blot analysis (inset) with an external probe, as indicated, resulted in a 7.5-kb band for the endogenous allele, whereas the knock-in cDNA alleles introduced a *Hind*III site and resulted in a 4.0-kb band.

RESULTS AND DISCUSSION

A kinase-dead variant of the ErbB-2/Neu receptor was created by oligonucleotide-directed PCR mutagenesis to generate a point mutation affecting lysine residue 757. This K757M alteration ablates the conserved ATP-binding lysine residue in the tyrosine kinase domain, resulting in its inability to phosphorylate its substrates (20, 21, 28). We have confirmed that disruption of this key amino acid results in ablation of ErbB-2-associated kinase activity and also inactivates the potent transforming activity of an oncogenic *erbB-2* mutant (data not shown).

To determine the functional importance of the ErbB-2 kinase activity *in vivo*, we generated a targeting vector in which the first coding exon of the endogenous *erbB-2* gene was replaced with either a wild-type *erbB-2* cDNA (*erbB-2* knock-in [KI]) (Fig. 1A) or a cDNA harboring the K747M mutation (*erbB-2* kinase-dead [KD]) (Fig. 1B). To facilitate recovery of targeted recombination events, a PGK-Neo expression cassette was inserted downstream of the inserted cDNA. The constructs were electroporated into R1 ES cells, and independent clonal lines were isolated and subjected to Southern blot analyses with an appropriate external probe to identify successful targeting events (Fig. 1).

After microinjection of several independently targeted ES cell lines into donor blastocysts, chimeric mice were obtained and bred to identify those that transmitted the mutant alleles through the germ line. *erbB-2*^{wt/KI} and *erbB-2*^{wt/KD} mice ap-

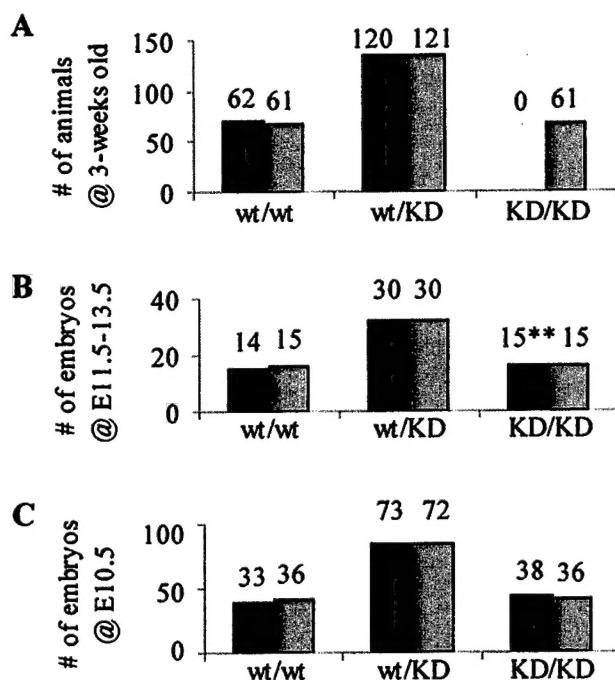


FIG. 2. Embryonic lethality at midgestation in kinase-dead mutants. Mendelian ratios from the progeny of heterozygous matings were determined and compared with the frequency of genotypes observed. (A) No homozygous *erbB-2*^{KD/KD} mutant animals were observed at weaning age (3 weeks old). (B) Observations at E11.5 to E13.5 revealed the expected number of mutant embryos; however, all *erbB-2*^{KD/KD} (**) embryos were being resorbed, and no heartbeat was detected. (C) At E10.5, all the embryos appeared healthy and were present in proportion with Mendelian frequencies. Solid bars, observed; shaded bars, expected.

peared normal and were fertile. *erbB-2*^{KI/KI} mice were also viable and were generated at the expected Mendelian ratios (data not shown). Thus, in contrast to the generation of *erbB-2*^{-/-} mice, in which exon 1 was replaced by a phosphoglycerate kinase (PGK)-neomycin resistance cassette (12), replacement of the first coding exon of *erbB-2* with a wild-type *erbB-2* cDNA rescued the embryonic lethality associated with disruption and inactivation of the *erbB-2* gene. Interestingly, no viable *erbB-2*^{KD/KD} mutant mice were observed in the litters generated from heterozygous matings that were subsequently genotyped at 3 weeks of age (Fig. 2A).

Since *erbB-2*^{-/-} embryos died at midgestation due to defects in heart development (12), we assessed whether we could detect viable embryos at E10.5 for our kinase-dead mutants. To accomplish this, timed matings between heterozygous animals were set up, and embryos were dissected from the uterus and observed. Embryo genotypes were determined by analysis of DNA isolated from their visceral yolk sacs. At E10.5, each possible genotype was present at the expected Mendelian frequencies (Fig. 2C), and all embryos were viable, possessed a heartbeat, and appeared normal in size. However, in mutant embryos the hearts were slightly enlarged and had irregular beats. Further observations at E11.5 to E13.5 (Fig. 2B) revealed that homozygous mutant *erbB-2*^{KD/KD} embryos, although present at the expected frequency, had no heartbeat and showed signs of resorption such as arrested growth, pale

color, and soft tissue. Thus, consistent with the embryonic lethality of *erbB-2*^{-/-} mutations, these observations confirmed that mutant embryos expressing the kinase-dead ErbB-2 receptor were dying in utero at midgestation, between E10.5 and E11.5.

To investigate the cause of embryonic lethality, we performed histological analyses of E10.5 embryos. Both *erbB-2*^{KI/KI} (Fig. 3A) and *erbB-2*^{wt/KD} (Fig. 3B) knock-in embryos were completely normal in their development of the heart trabeculae. In contrast, *erbB-2*^{KD/KD} mutant embryos clearly lacked development of ventricular trabeculae (Fig. 3C) that likely resulted in the observed reduction in embryonic blood flow. These results are strikingly consistent with the defects identified previously in *erbB-2*^{-/-} embryos as well as in *neuregulin*^{-/-} and *erbB-4*^{-/-} embryos (6, 12, 15). Thus, the cardiac defects seen in the kinase-dead mutants were directly attributable to a loss of ErbB-2's enzymatic tyrosine kinase activity.

We also examined whether the same peripheral nervous system abnormalities seen in *erbB-2*^{-/-} mutants were also sim-



FIG. 3. Defects in heart development in E10.5 mutant embryos. Parasagittal sections of (A) *erbB-2*^{KI/KI}, (B) *erbB-2*^{wt/KD}, and (C) *erbB-2*^{KD/KD} embryos at E10.5 were stained with hematoxylin and eosin. Although heartbeats were detected at the time of dissection, histological examinations of the hearts revealed a lack of trabeculae in the ventricles of *erbB-2*^{KD/KD} mutants but were present in heterozygous littermates and in age-matched *erbB-2*^{KI/KI} knock-in embryos. t, trabeculae; v, ventricle; ec, endocardial cushion; a, atrium.

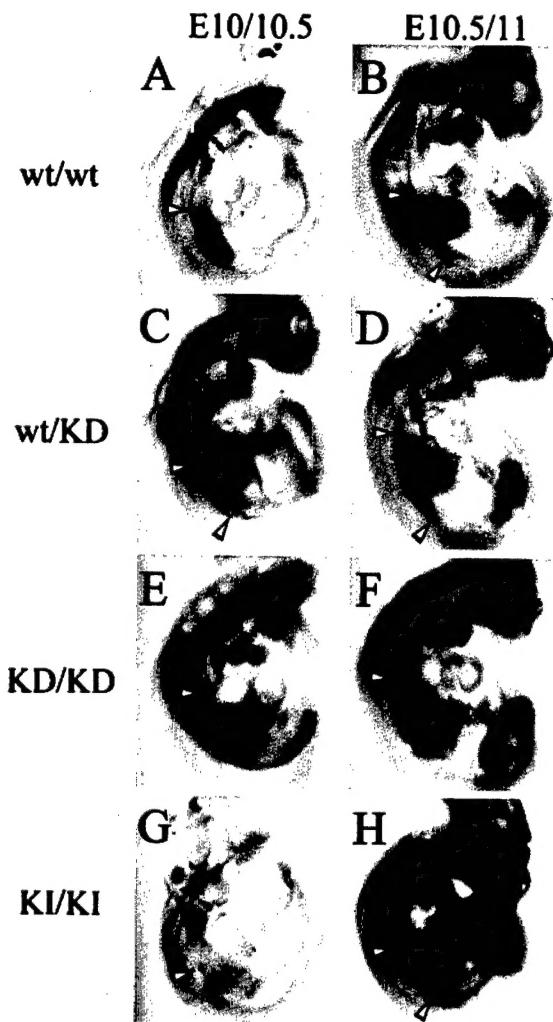


FIG. 4. Lack of sympathetic chain ganglia in kinase-dead mutants. Day E10 to E11 embryos were subjected to whole-mount *in situ* hybridization analysis using an antisense Phox2a riboprobe. Normal sympathetic chain ganglia development was present in (A and B) *erbB-2*^{wt/wt} embryos and (C and D) *erbB-2*^{wt/KD} heterozygous embryos, whereas (E and F) *erbB-2*^{KD/KD} homozygous mutants lacked proper development or they were delayed in (G and H) *erbB-2*^{KI/KI} embryos. The white arrowheads highlight the developing sympathetic chain ganglia.

ilarly affected by expression of the kinase-dead mutation. One important marker for peripheral neural tissues is the Phox2a transcription factor (16). To explore whether neural development was perturbed in the KD mice, embryos derived from E10.5 and E11.5 were subjected to whole-mount *in situ* analyses using an antisense Phox2a riboprobe (Fig. 4). In both *erbB-2*^{wt/wt} (Fig. 4A and B) and *erbB-2*^{wt/KD} (Fig. 4C and D) embryos, the sympathetic chain developed normally. However, in the E10.5 to 11 *erbB-2*^{KD/KD} homozygous embryos, only a weak Phox2a signal could be detected in the rostralmost regions of the sympathetic chain, a clear indication that this structure had either failed to initiate development or was incapable of developing in the kinase-dead genetic background (Fig. 4E and F).

Analyses of Phox2a expression in *erbB-2*^{KD/KD} (Fig. 4E) and *erbB-2*^{KI/KI} (Fig. 4G) embryos revealed that the sympathetic

chain appeared to be absent at E10 to 10.5 compared to age-matched wild-type and heterozygous embryos (compare Fig. 4G with Fig. 4A and C). However, by E10.5 to 11, a lengthy sympathetic chain had developed in the *erbB-2*^{KI/KI} homozygotes (Fig. 4H) but remained completely absent in the *erbB-2*^{KD/KD} embryos (Fig. 4F). These observations indicate that the catalytic activity of ErbB-2 is also essential for normal development of the primary sympathetic chain ganglia.

To confirm that these knock-in alleles expressed ErbB-2, we performed Western immunoblot (Fig. 5A) and RNase protection (Fig. 5B) analyses. As shown in Fig. 5B, the wild-type *erbB-2* KI allele and the kinase-dead allele expressed similar levels of *erbB-2* transcripts. Similarly, immunoblot detection for ErbB-2 also revealed comparable protein levels between the two knock-in mutants at E10.5 (Fig. 5A). The slightly lower levels of ErbB-2 seen in the *erbB-2*^{KD/KD} embryos relative to *erbB-2*^{KI/KI} embryos is likely due to and consistent with the loss of tissue structures in the kinase-dead mutants (Fig. 2 and 3), which would normally express significant levels of ErbB-2.

Interestingly, both the *erbB-2*^{KI/KI} and the *erbB-2*^{KD/KD} knock-in embryos expressed only 10 to 15% of the expected ErbB-2 protein as detected in *erbB-2*^{wt/wt} embryos (Fig. 5A, compare lanes 1 and 2 to lanes 3 to 6) despite expressing comparable levels of *erbB-2* transcripts (Fig. 5B), which may

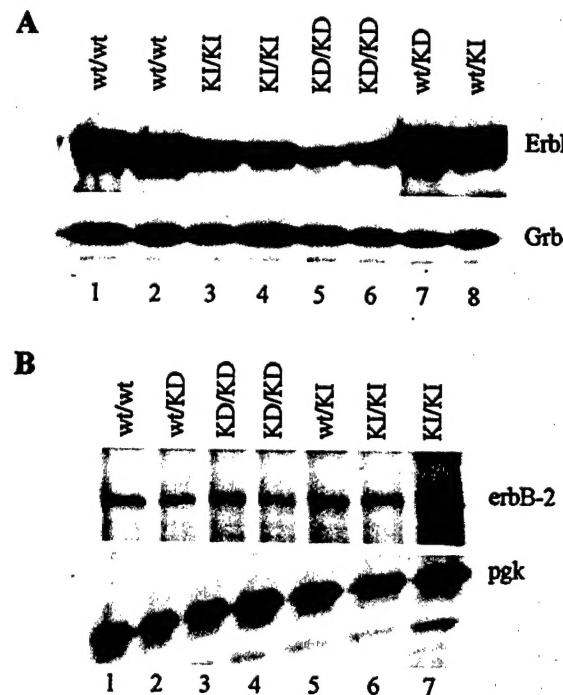


FIG. 5. Detection of ErbB-2 expression in E10.5 embryos. Expression of ErbB-2 generated from the cDNA insert in *erbB-2*^{KI/KI} and *erbB-2*^{KD/KD} embryos was determined. For (A) immunoblot analyses, total protein isolated from E10.5 embryos was subjected to SDS-PAGE and incubated with an anti-ErbB-2 antibody. Detection of Grb-2 (lower panel) protein was used to control for equal protein lysate quantification. (B) RNase protection assays with an antisense *erbB-2* riboprobe were used to detect *erbB-2* transcripts in total RNA isolated from *erbB-2*^{wt/wt} (lane 1), *erbB-2*^{KD/KD} (lanes 3 and 4), and *erbB-2*^{KI/KI} (lanes 6 and 7) embryos. A mouse PGK riboprobe (lower panel) was used as an internal control for equal sample loading in each lane.

reflect a requirement for splicing events for efficient transport and translation of the mRNA. In this regard it should be noted that embryos expressing both copies of the wild-type knock-in *erbB-2* allele display a 24-h delay in development of the sympathetic chain (Fig. 4), suggesting that reduced ErbB-2 levels may have a minor phenotypic consequence.

Our observations have important implications in understanding the role of *erbB-2* in promoting both normal cardiac and neural development. Previous studies have demonstrated that the integrity of ErbB-2, ErbB-4, or neuregulin is essential for the development of the cardiac trabecular extensions. Despite the presence of a functional ErbB-4 protein that could potentially transphosphorylate ErbB-2, our results further suggest that the catalytic activity of ErbB-2 alone is required to recapitulate the necessary signal transduction pathways leading to proper trabeculation.

In contrast to myocardial trabecula formation, ErbB-2 and ErbB-3 are thought to mediate the survival of neural crest cells, contributing to the development of the peripheral nervous system, since similar cranial nerve phenotypes were seen in mutant ErbB-2 and mutant ErbB-3 mice as well as in neuregulin mutants (12, 15, 22). Given that ErbB-3 is kinase defective and is completely dependent on its heterodimerization partners for its activation (8), inactivation of ErbB-2 catalytic activity would be expected to have profound effects on neural development. From these data, we can conclude that ErbB signaling is dependent on the kinase activity of ErbB-2 and that a kinase-dead ErbB-2 mutant acts essentially as a functionally null receptor.

In contrast to the embryonic lethality caused by inactivation of the catalytic activity of ErbB-2, mutants carrying a naturally occurring germ line mutation in the kinase domain of EGFR known as Waved-2 are completely viable and display only epithelial defects, such as a wavy hair phenotype (5, 14). Thus, unlike ErbB-2, other EGFR family members can presumably compensate for the severe impairment in EGFR catalytic activity. The difference between these phenotypes may reflect the hierarchical importance of ErbB-2 within the EGFR family signaling network. Alternatively, the difference in the phenotype in these strains may reflect the fact that the Waved-2 mutation has not completely ablated the catalytic activity of EGFR (14) and thus retains a higher degree of biological function.

Given the dominant-negative action of the kinase-dead ErbB-2 receptor expressed in vitro (20) it is surprising that mice heterozygous for the *erbB-2* KD allele failed to exhibit any obvious phenotype that might be expected of a *trans*-dominant inhibition of the remaining wild-type allele. One potential explanation for this observation is that the level of kinase-dead ErbB-2 is insufficient to interfere with the remaining endogenous wild-type ErbB-2 receptor. Indeed, the *erbB-2* KD allele produced only 10% of the expected ErbB-2 protein (Fig. 5). To exclude this possibility, we crossed *erbB-2* KI mice with *erbB-2* KD mice because both of these knock-in alleles expressed similar levels of ErbB-2. Phenotypic analyses of these crosses demonstrated that the *erbB-2* KD allele failed to exhibit a dominant-negative effect on the remaining *erbB-2* KI allele (data not shown). Thus, like the Waved-2 EGFR kinase mutation, the *erbB-2* KD allele fails to exhibit any discernible

dominant-negative effect on the remaining intact ErbB-2 receptor.

Studies with other receptor tyrosine kinases have concluded that the catalytic activity of receptor tyrosine kinases is dispensable for normal physiological function of the receptor. For example, the Flt-1 (vascular endothelial growth factor receptor) null mutation resulted in early embryonic lethality at E8.5 with disorganized blood vessels (4). However, mice expressing a kinase-deficient Flt-1 survived and showed normal angiogenesis (9), suggesting that other components of the receptor are more important to its functional role. In contrast to these observations, we have found that the catalytic activity of ErbB-2 is essential for embryonic development.

The embryonic lethality associated with the expression of the kinase-dead ErbB-2 is not likely due to inappropriate localization of the receptor, since it has previously been demonstrated that this mutant receptor is efficiently expressed on the cell surface (20). Given that ErbB-2 is the preferred heterodimerization partner for the other EGFR family members (7), the requirement for ErbB-2 catalytic function in vivo suggests that its catalytic activity is critical for EGFR family signaling. Future studies with these strains should allow identification of downstream targets of ErbB-2.

ACKNOWLEDGMENTS

R.C. and W.R.H contributed equally to this work.

We thank Margaret Hibbs and Ashley Dunn for providing genomic clones and Michael Rudnicki and Christo Goridis for providing plasmids used in this study. We also thank Brian Allore and Dinsdale Gooden at the MOBIX Central Facility, McMaster University, for automated DNA sequencing analysis and oligonucleotide synthesis.

This work was supported by a grant awarded to W.J.M. by the Medical Research Council of Canada. W.J.M. is a recipient of a Medical Research Council of Canada scientist award, and R.C. was supported by a studentship from the Cancer Research Society of Canada and a U.S. Army D.O.D. studentship.

REFERENCES

1. Britsch, S., L. Li, S. Kirchhoff, F. Theuring, V. Brinkmann, C. Birchmeier, and D. Riethmacher. 1998. The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. *Genes Dev.* 12:1825-1836.
2. Burden, S., and Y. Yarden. 1997. Neuregulins and their receptors: a versatile signaling module in organogenesis and oncogenesis. *Neuron* 18:847-855.
3. Carraway, K. L., and L. C. Cantley. 1994. A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. *Cell* 78: 5-8.
4. Fong, G. H., J. Rossant, M. Gertsenstein, and M. L. Breitman. 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376:66-70.
5. Fowler, K. J., F. Walker, W. Alexander, M. L. Hibbs, E. C. Nice, R. M. Bohmer, G. B. Mann, C. Thumwood, R. Maglito, and J. A. Danks. 1995. A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. *Proc. Natl. Acad. Sci. USA* 92:1465-1469.
6. Gassmann, M., F. Casagranda, D. Orioli, H. Simon, C. Lai, R. Klein, and G. Lemke. 1995. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* 378:390-394.
7. Graus-Porta, D., R. Beerli, J. M. Daly, and N. E. Hynes. 1997. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J.* 16:1647-1655.
8. Guy, P. M., J. V. Platko, L. C. Cantley, R. A. Cerione, and K. L. Carraway. 1994. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA* 91:8132-8136.
9. Hiratsuka, S., O. Minowa, J. Kuno, T. Noda, and M. Shibuya. 1998. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl. Acad. Sci. USA* 95:9349-9354.
10. Joyner, A. L. 1993. Gene targeting—a practical approach. Oxford University Press, New York, N.Y.
11. Karunagaran, D., E. Tzahar, R. R. Beerli, X. Chen, D. Graus-Porta, B. J.

Ratzkin, R., Seger, N. E., Hynes, and Y. Yarden. 1996. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J.* 15:254-264.

12. Lee, K. F., H. Simon, H. Chen, B. Bates, M. C. Hung, and C. Hauser. 1995. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* 378:394-398.

13. Lin, W., H. B. Sanchez, T. Deerinck, J. K. Morris, M. Ellisman, and K. F. Lee. 2000. Aberrant development of motor axons and neuromuscular synapses in erbB2-deficient mice. *Proc. Natl. Acad. Sci. USA* 97:1299-1304.

14. Luetke, N. C., H. K. Phillips, T. H. Qiu, N. G. Copeland, H. S. Earp, N. A. Jenkins, and D. C. Lee. 1994. The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. *Genes Dev.* 8:399-413.

15. Meyer, D., and C. Birchmeier. 1995. Multiple essential functions of neuregulin in development. *Nature* 378:386-390.

16. Morin, X., H. Cremer, M. R. Hirsch, R. P. Kapur, C. Goridis, and J. F. Brunet. 1997. Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene Phox2a. *Neuron* 18:411-423.

17. Morris, J. K., W. Lin, C. Hauser, Y. Marchuk, D. Getman, and K. F. Lee. 1999. Rescue of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. *Neuron* 23:273-283.

18. Pinkas-Kramarski, R., M. Shelly, B. C. Guarino, L. M. Wang, L. Lyass, I. Alroy, M. Alimandi, A. Kuo, J. D. Moyer, S. Lavi, M. Eisenstein, B. J. Ratzkin, R. Seger, S. S. Bacus, J. H. Pierce, G. C. Andrews, Y. Yarden, and M. Alamandi. 1998. ErbB tyrosine kinases and the two neuregulin families constitute a ligand-receptor network. *Mol. Cell. Biol.* 18:6090-6101.

19. Pinkas-Kramarski, R., L. Soussan, H. Waterman, G. Levkowitz, I. Alroy, L. Klapper, S. Lavi, R. Seger, B. J. Ratzkin, M. Sela, and Y. Yarden. 1996. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J.* 15:2452-2467.

20. Qian, X., W. C. Dougall, M. E. Hellman, and M. I. Greene. 1994. Kinase-deficient neu proteins suppress epidermal growth factor receptor function and abolish cell transformation. *Oncogene* 9:1507-1514.

21. Qian, X., C. M. LeVe, J. K. Freeman, W. C. Dougall, and M. I. Greene. 1994. Heterodimerization of epidermal growth factor receptor and wild-type or kinase-deficient Neu: a mechanism of interreceptor kinase activation and transphosphorylation. *Proc. Natl. Acad. Sci. USA* 91:1500-1504.

22. Riethmacher, D., E. Sonnenberg-Riethmacher, V. Brinkmann, T. Yamaai, G. R. Lewin, and C. Birchmeier. 1997. Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* 389:725-730.

23. Siegel, P. M., D. L. Dankort, W. R. Hardy, and W. J. Muller. 1994. Novel activating mutations in the *neu* proto-oncogene involved in induction of mammary tumors. *Mol. Cell. Biol.* 14:7068-7077.

24. Siegel, P. M., E. D. Ryan, R. D. Cardiff, and W. J. Muller. 1999. Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. *EMBO J.* 18:2149-2164.

25. Valarce, I., J. P. Tissier-Seta, M. R. Hirsch, S. Martinez, C. Goridis, and J. F. Brunet. 1993. The mouse homeodomain protein Phox2 regulates Ncam promoter activity in concert with Cux/CDP and is a putative determinant of neurotransmitter phenotype. *Development* 119:881-896.

26. Wallasch, C., F. U. Weiss, G. Niederfellner, B. Jallal, W. Issing, and A. Ullrich. 1995. Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. *EMBO J.* 14:4267-4275.

27. Webster, M. A., J. N. Hutchinson, M. J. Rauh, S. K. Muthuswamy, M. Anton, C. G. Tortorice, R. D. Cardiff, F. L. Graham, J. A. Hassell, and W. J. Muller. 1998. Requirement for both Shc and phosphatidylinositol 3' kinase signaling pathways in polyomavirus middle T-mediated mammary tumorigenesis. *Mol. Cell. Biol.* 18:2344-2359.

28. Weiner, D. B., Y. Kokai, T. Wada, J. A. Cohen, W. V. Williams, and M. I. Greene. 1989. Linkage of tyrosine kinase activity with transforming ability of the p185neu oncoprotein. *Oncogene* 4:1175-1183.

29. Wilkinson, D. G., and M. A. Nieto. 1993. Detection of messenger RNA by *in situ* hybridization to tissue sections and whole mounts. *Methods Enzymol.* 225:361-373.

30. Yarden, Y., and A. Ullrich. 1988. Growth factor receptor tyrosine kinases. *Annu. Rev. Biochem.* 57:443-478.